Biopolymers

Edited by A. Steinbüchel

Volume 3a
Polyesters I
Biological Systems and
Biotechnological Production

Edited by Y. Doi and A. Steinbüchel

WILEY-VCH

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Library of Congress Card No: applied for
British Library Cataloguing-in-Publication Data: A catalogue record for this book is available from the British Library

Die Deutsche Bibliothek –
CIP-Cataloguing-in-Publication Data
A catalogue record for this publication is available from Die Deutsche Bibliothek

Printed in the Federal Republic of Germany
Printed on acid-free paper.

Composition, Printing, and Bookbinding:
Konrad Triltsch
Print und digitale Medien GmbH,
Ochsenfurt-Hohestadt

ISBN 3-527-30224-7

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10

Fermentative Production of Medium-chain-length Poly(3-hydroxyalkanoate)

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MCL	medium-chain-length
Poly(3HA)	poly(3-hydroxyalkanoate)
Poly(3HB)	poly(3-hydroxybutyrate)
Poly(3HB-*co*-3HV)	poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
PSA	pressure-sensitive adhesive
SCL	short-chain-length

1 Introduction

Medium-chain-length poly(3-hydroxyalkanoate) (MCL-Poly(3HA)) forms a large and versatile family of polyesters produced by various bacteria as storage. MCL-Poly(3HA)s are receiving considerable attention because of their potential as renewable and biodegradable plastics, and the monomers as a source of chiral synthons. A wide range of substituted hydroxyalkanoic acids can be incorporated into these polyesters in biotechnological processes. Various fermentation strategies have been developed and optimized in order to control the monomer composition of the polymer, enabling the tailoring of the material properties and the production of MCL-Poly(3HA)s in an economically efficient manner. Production processes of MCL-Poly(3HA) are presented in comparison to alternative production strategies. Furthermore, biosynthesis of MCL-Poly(3HA)s, including 'functionalized' Poly(3HA)s, is discussed.

2 Historical Outline

The first example of microbial Poly(3HA)s to be discovered was polyhydroxybutyrate (Poly(3HB)) in 1926 (Lemoigne, 1926). Since then Poly(3HB) accumulation was found in various microorganisms, representatives of Gram-negative and Gram-positive species (i.e. autotrophs, heterotrophs, phototrophs, aerobes, and anaerobes), and archaebacteria (as reviewed elsewhere: Steinbüchel, 1991; Lee, 1996; Sasikala and Ramana, 1996).

The discovery of a polyester consisting mainly of hydroxyoctanoate monomers by de Smet et al. (1983) was the first example of a new group, the so called MCL-Poly(3HA)s, which can contain a wide variety of different monomers.

The MCL-Poly(3HA)s are of interest for specific uses, where the chirality and elastomeric properties of the polymers are important. In addition, the monomers of Poly(3HA)s that contain different functional groups in their side chain are receiving more and more attention as source of chiral synthons (Ohashi and Hasegawa, 1992; Walbiel et al., 1992). In this report we will focus on microbial production of these polyesters by fermentation and present economic considerations.

3 Occurrence

MCL-Poly(3HA) production is restricted to fluorescent Pseudomonads belonging to rRNA-homology group I (Hofmann et al., 1989). Members of this group are, amongst others, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas oleovorans*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas testosteroni*, and *Pseudomonas putida*. MCL-Poly(3HA) is not just one single polymer, but a family of biopolyesters, which differ with respect to monomer composition. To date, more than 100 different monomers were found in the polymers (Steinbüchel and Valentin, 1995). Among these are 3-hydroxy acids of 6–16 carbon atoms with a large variety of saturated, unsaturated, straight, or branched chains containing aliphatic or aromatic side groups. Furthermore, monomers with various different functional groups in the side chain such as halogen atoms, hydroxy-, epoxy-, cyano, carboxyl-, phenoxy-, cyanophenoxy-, nitrophenoxy-, and esterified carboxyl groups have been introduced into MCL-Poly(3HA)s (for review, see: Lenz et al., 1992; Steinbüchel and Valentin, 1995; Sasikala and Ramana, 1996). The 3-hydroxyalkanoic acid monomer units in these microbial polyesters are all in the *R*-configuration due to the stereospecificity of biosynthetic enzymes.

The molecular weights of the polymers range from 2×10^5 to 3×10^6, depending on the specific polymer, the microorganism, and the growth conditions.

4 Functions

MCL-Poly(3HA)s function as a reserve materials for carbon and energy. They are formed when an excess carbon source is present.

Because MCL-Poly(3HA) is a polymer, a large amount of reserve material can be stored without affecting the osmotic pressure of the cell. When the supply of the carbon source becomes limiting, Poly(3HA) can be degraded by intracellular depolymerases and subsequently metabolized as carbon and energy source (Merrick and Doudoroff, 1964). The ability to convert excess substrate in the environment to reserve material is an advantage in the competition for survival because it limits the availability of the substrate for other microorganisms.

Another possible function of MCL-Poly(3HA) is detoxification. Substrates such as alkanes, alkanols, and fatty acids are toxic to microorganisms at low concentrations. Fast removal of these substrates from the environment by conversion to MCL-Poly(3HA) would improve the viability of the microorganism (Kranz et al., 1997).

Apparently, different kinds of Poly(3HA)s have been developed during evolution. This makes one wonder what the functional differences between these Poly(3HA)s are. The calculated energetic efficiencies of MCL-Poly(3HA) and SCL-Poly(3HA) are compared below.

MCL-Poly(3HA) is especially effective as a storage material when aliphatic substrates are used as a carbon source. For example, the conversion of decanoic acid into acetyl-CoA via MCL-Poly(3HA) [poly(3-hydroxydecanoate)] costs only 1 additional ATP compared to the direct conversion of decanoic acid to acetyl-CoA, assuming that the Poly(3HA) monomers are activated after depolymerization by means of a synthetase (Figure 1a). If SCL-Poly(3HA) [Poly(3HB)] is the storage material, 2.5 ATP has to be invested (Figure 1b). Also the efficiency in storage of the reducing power of MCL-Poly(3HA) with aliphatic substrates is higher. The conversion of decanoic acid into 3-hydroxydecanoic acid generates only 1 FADH; the remaining reducing power is stored in the polymer (Figure 1a). The conversion of decanoic acid to 3-hydroxybutyric acid, on the other hand, generates more reducing power equivalents, 3 NADH and 4 FADH, resulting in a lower reducing-power storage capacity (Figure 1b).

SCL-Poly(3HA)s, on the other hand, are more efficient storage materials when carbohydrates are used as a carbon source. This is caused by the fact that production of MCL-Poly(3HA) by fatty acid synthesis requires more ATP and reducing equivalents than the degradation of MCL-Poly(3HA) by β-oxidation generates (Figures 1c and d).

Thus, MCL-Poly(3HA) is the more efficient storage material when aliphatic substrates are degraded by the β-oxidation pathway, whereas SCL-Poly(3HA) are more efficient with other substrates.

5 Biochemistry

The material properties of MCL-Poly(3HA) can be programmed during the fermentation phase. The most important tool to control the material properties is the monomer composition. The monomer composition of MCL-Poly(3HA) can be varied by using different substrates. The conversion of these substrates is specific for the substrate used and the metabolic pathway involved.

5.1 β-Oxidation

Lageveen et al. (1988) showed that the monomer composition of aliphatic saturated MCL-Poly(3HA) produced by *P. oleovorans* depended on the type of *n*-alkane used. It appeared that the *n*-alkanes were degraded by the subsequent removal of C2 units and therefore it was proposed that the β-oxida-

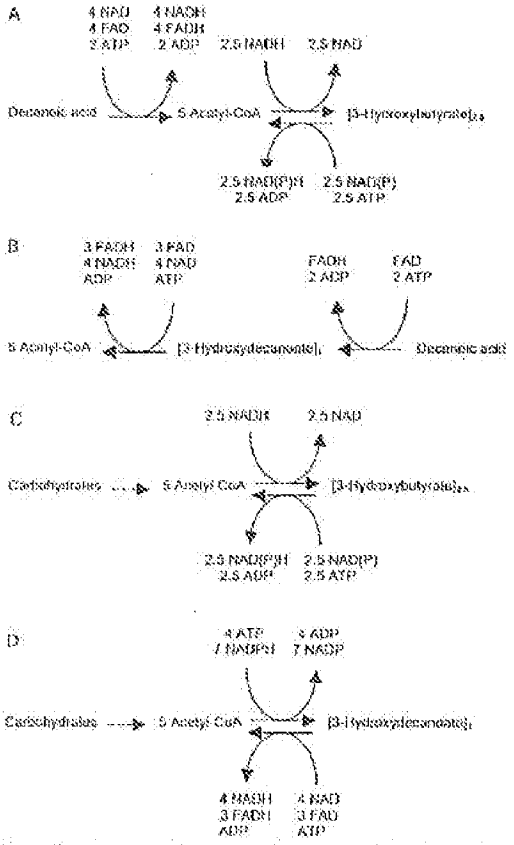

Fig. 1 Schematic overview of the energetics of the conversion of carbohydrates and fatty acids into SCL-PHA (PHB) and MCL-PHA [poly(3-hydroxydecanoate)].

Tab. 3 Monomer composition of the MCL-poly(3HA)s produced by *P. oleovorans* grown on n-alkanes as the sole carbon and energy source (Preusting et al., 1990)

C source	C_6	C_8	C_8	C_{10}	C_{10}	C_{10}
n-Hexane			81.1 ± 0.5	< 1.0	12.6 ± 0.2	< 1.0	4.9 ± 0.1
n-Heptane		2.5 ± 0.1		97.5 ± 0.1	< 1.0	< 1.0	
n-Octane	< 1.0		12.2 ± 0.2		67.8 ± 0.7		< 1.0
n-Nonane		2.1 ± 0.1		80.6 ± 0.3	5.4 ± 0.2	11.7 ± 0.4	
n-Decane	< 1.0		11.1 ± 0.1	1.1 ± 0.1	63.8 ± 0.2	1.2 ± 0.1	20.8 ± 0.2

C6: 3-hydroxyhexanoic; C8: 3-hydroxyoctanoic; C8': 3-hydroxy-5-cis-octenoic; C10: 3-hydroxydecanoic; C10': 3-hydroxy-7-cis-decenoic; C10'': 3-hydroxy-5-cis-decenoic.

three pathway was involved in MCL-Poly(3HA) biosynthesis. Preusting et al. (1990) confirmed these results, but also showed that octane as substrate 3-hydroxyoctanoate and 3-hydroxydecanoate were produced, indicating that also other pathways were involved in MCL-Poly(3HA) biosynthesis (Table 3).

Comparable results were found with MCL-Poly(3HA) production by *P. putida* KT2442 using fatty acids as substrate (Huijberts et al., 1992). Studies with ^{13}C-labeled decanoic acid and inhibitors of β-oxidation and fatty acid synthesis showed that this substrate was converted into MCL-Poly(3HA) by the β-oxidation pathway exclusively.

5.2
Fatty Acid Synthesis

Experiments with ^{13}C-labeled hexanoic acid as a substrate for *P. putida* KT2442 showed that three pathways are involved in its conversion into MCL-Poly(3HA). Hexanoic acid can be incorporated directly into MCL-Poly(3HA) as 3-hydroxyhexanoic acid after both a cycle of β-oxidation. Further, it appeared that part of the hexanoic acid is partly or fully degraded by the β-oxidation cycle and that the generated acetyl-CoA is used for *de novo* fatty acid synthesis to produce C8 to C14 monomers. Also, the presence of unsaturated monomers suggests that *de novo* fatty acid synthesis is active. There was also evidence that hexanoic acid was elongated to 3-hydroxyoctanoic acid (Huijberts et al., 1994).

Non-MCL-Poly(3HA)-related substrates like glucose, fructose, and glycerol can be converted into MCL-Poly(3HA) (Haywood et al., 1990; Timm and Steinbüchel, 1990; Huijberts et al., 1992). This MCL-Poly(3HA) consists mainly of C8 and C10 monomers. The fatty acid synthesis inhibitor cerulenin stopped MCL-Poly(3HA) production. Also, the temperature-dependent presence of unsaturated monomers, which resembled the temperature-dependent production of unsaturated fatty acids, indicated that carbohydrates can be transformed into MCL-Poly(3HA) by means of fatty acid synthesis.

5.3
Unsaturated Fatty Acids

The 3-hydroxy fatty acids with functional groups can be incorporated in MCL-Poly(3HA). In particular, unsaturated 3-hydroxy fatty acids are readily integrated in MCL-Poly(3HA) by using aliphatic unsaturated substrates for growth. De Waard et al. (1993) used oleic acid and linoleic acid as substrates for MCL-Poly(3HA) production by *P. putida* KT2442. It was found that oleic acid was degraded via the enoyl-CoA isomerase-dependent route and linoleic acid via the dienoyl-CoA reductase-dependent route.

6
Physiology and Process Development

Process development of microbial MCL-Poly(3HA) production has been focussed on optimization of such process parameters as yield, productivity, and Poly(3HA) content of the biomass; on the dilemma of how to dose toxic substrates that are difficult to measure on line at substrate excess concentrations; and on the control of the monomer composition and material characteristics of MCL-Poly(3HA) by adjustment of the feed composition.

6.1
Fermentation Process Development

Fermentation process development has recently been reviewed by Kessler et al. (2001). Of the fluorescent Pseudomonads, two species have been studied most extensively for MCL-Poly(3HA) production: *P. oleovorans* and *P. putida*. These microorganisms show a striking physiological dissimilarity with respect to MCL-Poly(3HA) production.

P. oleovorans is able to use alkanes and alkenes as a substrate due to the presence of the OCTT plasmid (Kok, 1988), whereas *P. putida* is not able to oxidize alkanes/alkenes. *P. putida*, however, can, in contrast to *P. oleovorans*, use carbohydrates, such as glucose and fructose, for the production of MCL-Poly(3HA) (Haywood et al., 1990; Timm and Steinbüchel, 1990; Huijberts et al., 1992).

P. putida is able to produce MCL-Poly(3-HA) during exponential growth, when all nutrients are available in sufficient amounts. MCL-Poly(3HA) production in *P. oleovorans*, however, only occurs when the concentration of one of the nutrients is limiting growth.

6.1.1
P. oleovorans

The development of fermentation processes for the production of MCL-Poly(3HA) started with the experiments carried out by Preusting et al. (1993a). *P. oleovorans* was grown in two-phase fed-batch cultivations. The two phases consisted of a watery phase containing mineral nutrients and an organic phase of octane. Using an organic phase is convenient because this results, without extra addition during the process, in a constant availability of the carbon source for the microorganisms in the watery phase. The feed rate of the growth-limiting substrate was constant. After an initial batch period nitrogen became limited. A biomass concentration of 37.1 g L^{-1} was reached in 48 h, containing 33% of MCL-Poly(3HA), resulting in a productivity of 0.25 g L^{-1} h^{-1}.

With a comparable set-up, continuous cultivations were performed (Preusting et al., 1993b). The optimal growth rate was 0.05 h^{-1}. The maximum productivity was 0.58 g L^{-1} h^{-1}, with a maximum biomass concentration of 11.6 g L^{-1}. Compared with the fed-batch experiments, however, the MCL-Poly(3HA) content decreased to 20%. The restricted retention time of the microorganism in the culture appears to limit the maximal attainable Poly(3HA) content.

The medium composition used in the fed-batch process was optimized resulting in cell densities near 100 g L^{-1}. By applying an exponential feed rate resulting in a growth rate of 0.05 h^{-1}, the maximal biomass concentration increased further to 112 g L^{-1}, with a biomass productivity of 1.8 g L^{-1} h^{-1}. The MCL-Poly(3HA) productivity, however, was low, 0.34 g L^{-1} h^{-1}, caused by a steady decrease of the MCL-Poly(3HA) content during the last part of the fermentation

[Huisenberg, 1997]. When this optimized medium composition was used in the chemostat set-up described above, a maximum biomass concentration of 18 g L^{-1} was reached. The MCL-Poly(3HA) content, however, remained low at approximately 18% (Huisenberg, 1997). It is still unclear what causes these low MCL-Poly(3HA) contents.

In order to develop a more efficient MCL-Poly(3HA) production process, a two-stage continuous culture system was set-up. In the first phase, biomass was produced; in the second stage, MCL-Poly(3HA) was synthesized in the absence of a nitrogen source. A maximum polymer content of 63% was reached, at a productivity of 1.46 g L^{-1} h^{-1}. This polymer content is the highest reported for MCL-Poly(3HA) to date [Huisenberg, 1997; Jung et al., submitted].

Fed-batch fermentations with *P. oleovorans* have been carried out using octanol and octanoate as substrate [Lee and Chang, 1995]. Pure oxygen was used to ensure high oxygen transfer rates. With octanoate as substrate, 44.8 g L^{-1} biomass with a cellular Poly(3HA) content of 57% and a productivity of 0.35 g L^{-1} were reached. Higher biomass concentrations could not be achieved due to accumulation of the toxic octanoate.

6.3.2
P. putida

In parallel, MCL-Poly(3HA) production processes with *P. putida* have been developed. *P. putida* does, in contrast to *P. oleovorans*, not have to be grown under nutrient-limited conditions to produce MCL-Poly(3HA). Another difference between both organisms is that *P. putida* is not able to use alkanes or alkenes as substrate. Instead, fatty acids have been used as a carbon source. These fatty acids cannot, however, be used as a second phase during fermentation because the resulting high concentrations of the fatty acids are toxic. In high-cell-density continuous culture *P. putida* has been grown to 30 g L^{-1} and 23% MCL-Poly(3HA) with oleic acid as substrate, corresponding to a productivity of 0.97 g L^{-1} h^{-1} (Huijberts and Eggink, 1996).

To perform fed-batch experiments with *P. putida* a method had to be developed to prevent carbon limitation and to prevent a build up of the concentration of the fatty acids to inhibitory levels. High-performance liquid chromatography methods to measure the concentration of aliphatic substrates have been reported, also for octanoic acid (Kim et al., 1996, 1997), but these are not suitable for the detection of long chain fatty acids in a watery phase due to their low solubility. Instead a method was developed in which the fatty acids were added pulse-wise to the cultures (Huijberts, 1995; Weusthuis et al., 1997). Substrate exhaustion was detected by a sudden increase in dissolved oxygen tension and this signal was used to pulse a further amount of fatty acids into the fermenter. In this way the time the culture was carbon limited could be minimized and the maximum concentration of fatty acids could be controlled to prevent toxic levels. With coconut oil fatty acids as substrate, a maximal biomass concentration of 11 g L^{-1} after 36 h was reached containing 59% of MCL-Poly(3HA) resulting in a maximal productivity of 2.3 g MCL-Poly(3HA) L^{-1} h^{-1} (Figure 3). This is the highest productivity reported to date. The same experiment has also been performed with fatty acids derived from linseed oil, coconut oil, tall oil, rape seed oil, and mixtures of these with comparable results. This allows the production of MCL-Poly(3HA) with various monomer compositions.

These results show that, up to now, fed-batch cultivation is the method of choice for *P. putida*. The low Poly(3HA) content of the

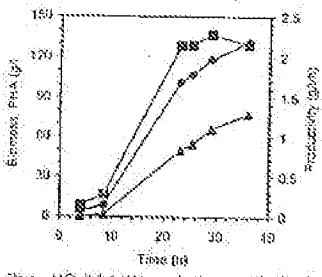

Fig. 3 MCL-Poly(3HA) production as a fed-batch fermentation with *P. putida* KT2442 and coconut oil fatty acids as substrate. ◆: Biomass; ▲: MCL-Poly(3HA); ■: MCL-Poly(3HA) productivity.

biomass grown in chemostat cultures renders this cultivation method unsuitable for large-scale production.

6.4
Control of MCL-Poly(3HA) Monomer Composition

Intermediates of the β-oxidation pathway are incorporated in MCL-Poly(3HA), as shown in Section 5.3. Both the β-oxidation pathway and the enzymes involved in MCL-Poly(3-HA) formation are highly aspecific. This opens the possibility to control the monomer composition of MCL-Poly(3HA) and to program material characteristics.

6.4.1
Length and Unsaturation of MCL-Poly(3HA) Monomers

With oleic acid, mono-unsaturated monomers were incorporated in MCL-Poly(3HA); with linoleic acid, 2-fold unsaturated monomers were also detected (De Waard et al., 1993). Casas et al. (1992) used hydrolyzed linseed oil as substrate for *P. putida* KT2442. The presence of the 3-fold unsaturated linoleic acid led to the incorporation of C14:3 and C16:3 3-hydroxy fatty acids in MCL-Poly(3HA). This was the first time that C16:3 3-hydroxy fatty acids were found to be also incorporated in MCL-Poly(3HA).

Furthermore, MCL-Poly(3HA)s were produced from free fatty acid mixtures derived from industrial byproducts, such as tall oil fatty acids, which showed an interesting potential as low-cost renewable resources. Isolation and analysis of the polymer allowed the identification of 16 different saturated, mono-unsaturated, and di-unsaturated monomers (Kellerhals, 1999). Except for the presence of diene-containing monomers and the large number of minor components, the monomer composition of the fatty acid mixture-derived MCL-Poly(3HA) did not differ significantly from oleic acid-derived Poly(3HA)s.

When a mixture of fatty acids or hydrocarbons is used as substrate, all compounds are simultaneously used for growth and MCL-Poly(3HA) production. In that way it is possible to control the monomer composition (length of carbon chain of monomer, number and type of unsaturations, and other functionalities) of MCL-Poly(3HA) to some extent, enabling the tailoring of the material properties to meet the demands of specific applications (Figure 3).

6.4.2
Production of MCL-Poly(3HA)s with other Functionalities

It has been shown that more than 60 different monomers can be incorporated into Poly(3HA) by Pseudomonads (Steinbüchel and Valentin, 1995). Poly(3HA)s containing a functional group in their side chain are generally called 'functional' Poly(3HA)s.

One strategy to produce MCL-Poly(3HA)s with a certain monomer content is co-feeding of two different substrates in a certain ratio. In principle three types of

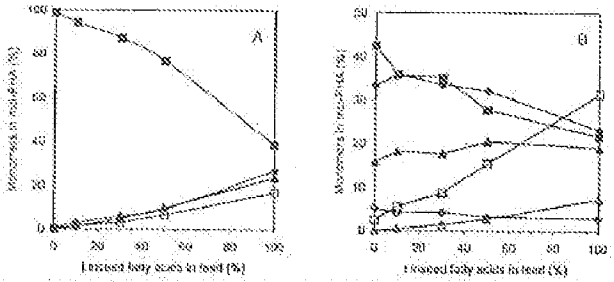

Fig. 3 The effect of the fatty acid composition of the substrate fed to a high-cell density fed-batch cultivation of *P. putida* KT2442 on the degree of unsaturation (A) and the carbon chain length of the MCL-Poly(3HA) monomers. The substrates used were mixtures of coconut oil fatty acids and fatted oil fatty acids. (A) ●: saturated; ▲: monounsaturated; □: 2-fold unsaturated; ▽: 3-fold unsaturated. (B) □: 3-hydroxy C6 fatty acids; ▽: 3-hydroxy C8 fatty acids; ●: 3-hydroxy C10 fatty acids; ▲: 3-hydroxy C12 fatty acids; ■: 3-hydroxy C14 fatty acids; ◇: 3-hydroxy C16 fatty acids.

substrates have to be differentiated: (1) substrates which support cell growth and Poly(3HA) production, (2) substrates which support growth but not Poly(3HA) production, and (3) substrates which do not support growth but do support Poly(3HA) production (Lenz et al., 1992). Therefore, dependent on the type of substrate, different cultivation processes and feeding strategies have to be used.

It has been shown that application of carbon source mixtures such as octanoic/octanoate (Durner, 1998) or glucose/octanoic acid (Kim et al., 1996, 1997) which support cell growth and Poly(3HA) production, respectively, are utilized simultaneously in batch cultures, and that fatty acids were used for Poly(3HA) synthesis and carbohydrates were dissimilated to supply the reducing equivalents. In general, support of bacterial cell growth by one substrate and Poly(3HA) formation, especially the incorporation of specific monomers, by the other substrate is a widespread technique for the production of 'functionalized' polymers (e.g. Scholz et al., 1991; de Koning et al., 1994; Hori et al., 1994; Kim, G. Y., et al., 1995, 1996; Curley et al., 1996a; Cross et al., 1996; Nomi et al., 1996). Another possibility is to perform a two-stage cultivation process. In the first stage bacterial cell mass is produced and in the second stage Poly(3HA)-forming substrates are added to the culture, as has been reported for the production of Poly(3HA) containing multi-fluorinated, cyano, or nitrophenoxy side chain substituents (Kim, G. Y., et al., 1995, 1996).

In many cases co-feeding strategies are not only used to produce specific random copolymers – even block polymers or polymer blends can be obtained. Growth of *P. oleovorans* or *P. putida* on a mixture of 5-phenylvaleric acid (or other arylalkyl acids) and nonanoic acid results in a homopolymer poly-3-hydroxy-5-phenylvalerate, and a random copolymer consisting of 3-hydroxynonanoate and 3-hydroxyheptanoate (Kim et al., 1991; Curley et al., 1996b; Hazer et al., 1996). It has been shown that both types of polyesters occur in the same granule (Curley, 1996b). Interestingly, it has even been proposed that by sequential feeding of nonanoic acid and 10-undecenoic acid, a physical mixture of two different polymers is produced; however, with small amounts of Poly(3HA) containing repeating units from both substrates (Kim, Y. B., et al., 1997), whereas co-feeding of octanoate and cyanophenoxyalkanoates resulted in Poly(3HA) block polymers containing chain segments that are enriched in 3-hydroxycyanophenoxyalkanoate monomers (Cross et al., 1996).

Production of MCL-Poly(3HA)s from toxic organic solvents requires other cultivation strategies. A cultivation method was developed to improve growth of *P. oleovorans* on toxic organic solvents, such as 1-hexene. This method includes dilution of 1-hexene with a non-metabolizable second organic phase to lower the toxic effect of the apolar carbon source and a long-term chemostat enrichment culture to increase the solvent tolerance and the specific growth rate (Jung et al., submitted). Furthermore, application of dual-carbon/nitrogen-limited conditions for cell growth and Poly(3HA) production on volatile and toxic substrates resulted in decreased cell lysis, side product formation, and biosurfactant production, and therefore higher cell and Poly(3HA) yields (Jung et al., submitted).

6.3 Oxygen Transfer and Heat Production

The importance of a good oxygen transfer is stressed in many publications concerning the biotechnological production of MCL-Poly(3HA) (e.g. Lee and Chang, 1995; Huijberts, 1996; Hazenberg, 1997). Oxygen uptake rates as high as 280 (Hazenberg, 1992) and 230 (Huijberts, 1996) mmol L^{-1} h^{-1} have been described. By using reduced substrates as alkanes and fatty acids a lot of oxygen is necessary for the conversion of these aliphatic substrates into MCL-Poly(3HA) and, especially, into biomass.

In the fed-batch production process of MCL-Poly(3HA) by *P. putida* KT2442 as described above, the oxygen transfer limits the productivity and final biomass concentration. In addition, the Poly(3HA) content of biomass is positively affected by high oxygen transfer rates. At the end of the cultivation biomass production stops because all oxygen is used for maintenance processes (Figure 4a). The productivity of biomass and MCL Poly(3HA), but also the final biomass concentration, final Poly(3-HA) concentration, and maximal Poly(3HA) content of the biomass, depend on the maximal oxygen transfer rate during the fermentation (Figure 4b).

The high oxygen transfer rates reached in laboratory fermentors are not easily reached at a production scale. The heat development by excessive oxygen consumption will also result in cooling problems. Methods to reduce the oxygen consumption rate have been mentioned. There are two promising possibilities. First, by increasing the Poly(3-HA) content of the biomass (thereby decreasing the amount of biomass) the oxygen consumption can be limited. Second, by using oxidized co-substrates the oxygen consumption can be decreased. Durner (1998) showed that citrate and octanoic acid can be used simultaneously in batch cultures of *P. oleovorans* and Kim et al. (1996, 1997) demonstrated the same for the combination of glucose and octanoic acid by high-cell-density fed-batch processes of *P. putida*. These findings indicate that Pseudomonads are able to use different unrelated substrates simultaneously even under carbon excess conditions.

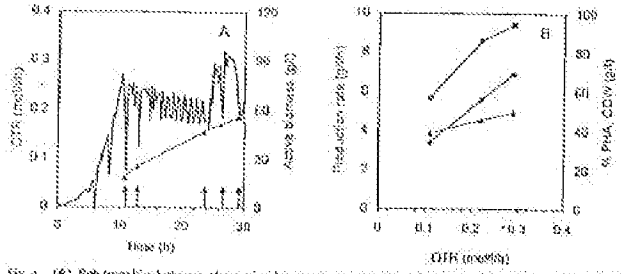

Fig. 4 (A) Relationship between the active biomass concentration (product of biomass without MCL-Poly(3HA), ●, endogenous respiration for maintenance purposes and the oxygen transfer rate (OTR, —) in a high cell density fed-batch fermentation of *P. putida* KT2442 cultivated on coconut oil fatty acids. The endogenous respiration was estimated at several time intervals by measuring the transient decrease in oxygen uptake rate going from substrate excess conditions to conditions where the substrate was fully consumed (indicated by arrows). There is a clear correlation between the endogenous respiration rate and the concentration of active biomass resulting in a complete utilization of the transferred oxygen for maintenance purposes after 24 h. After 24 h the oxygen transfer rate was increased by using a higher aeration rate, resulting in further growth. (B) The effect of the maximum oxygen transfer rate on the biomass concentration (●), MCL-Poly(3HA) content (▲) and biomass production rate (▲) of high cell-density fed-batch fermentations of *P. putida* KT2442 using coconut oil fatty acids as substrate. The production rate is defined as the increase in biomass concentration during the linear growth phase divided by the duration of the linear growth phase.

6.6
Byproducts

Fluorescent Pseudomonads are known to produce several byproducts. Siderophores as pyoverdines (Nüdmann and Meyer, 1988) and pyochelin (Cox et al., 1981), antibiotics such as phenazine derivatives (Thomashow and Weller, 1988), pyrrolnitrin, pyoluteorin, and 2,4 diacetyl phloroglucinol (Dowling and O'Gara, 1994), and surfactants such as rhamnolipids (Fiechter, 1992) can be produced. No attempts have been made to determine the presence of these compounds in fermentation broth used for MCL-Poly(3-HA) production. Fluorescence and extensive foaming of fermentation broth samples of *P. putida* cultivated in high cell density fed-batch fermentations with fatty acids as substrate, however, indicate that in cultures used for MCL-Poly(3HA) production by-products are also formed and that they influence the fermentation process (unpublished results).

7
Molecular Genetics

An alternative and additional approach to increase Poly(3HA) yield, productivity, and Poly(3HA) content of biomass has been by genetic modification. Poly(3HA) production of both recombinant fluorescent Pseudomonads and *Escherichia coli* has been studied.

7.1
Recombinant Pseudomonads

P. oleovorans GPo1, *P. putida* KT2442, and *P. aeruginosa* PAO1 are the best studied MCL-Poly(3HA)-producing strains on a genetic level. These bacteria contain two Poly(3HA) polymerases (also called Poly(3HA) synthases) encoded by *phaC1* and *phaC2* of the *pha* gene cluster (Huisman et al., 1991; Timm and Steinbüchel, 1992). It has been shown for *P. oleovorans* that the two Poly(3HA) polymerases have a small difference in substrate specificity (Huisman et al., 1992). Moreover, it was demonstrated that both polymerases are functional proteins which are able to catalyze Poly(3HA) formation independently from each other, i.e. only one of the polymerase encoding genes is enough to produce MCL-Poly(3HA) in heterologous hosts (Huisman et al., 1992; Langenbach et al., 1997; Ren, 1997; Mittendorf et al., 1998). Introduction of additional copies of the Poly(3HA) polymerases-encoding genes resulted in a nearly 2-fold increase in Poly(3HA) when the strains were cultivated under non-limited conditions (Kraak et al., 1997). However, no significant increase in Poly(3HA) accumulation was observed when the recombinants were cultivated under nutrient-limited conditions. The only effect of additional copies of the Poly(3HA) polymerase encoding genes was a slight change in the monomer composition of the polymer and a decrease in its molecular weight (Huisman et al., 1992; Kraak et al., 1997). Furthermore, it has been reported that GPp120, a chemical mutant of *P. putida* KT2442, produces higher levels of Poly(3-HA) in shaking flask experiments than the parental strain and that the mutant did not show any downregulation of Poly(3HA) formation under non-limiting conditions (Ren et al., 1998). However, a detailed analysis of the mutant showed that due to the reduced growth rate, the Poly(3HA) yield was only half of that found for the wild-type strain (unpublished results). In conclusion, all recombinant *Pseudomonas* strains or mutants tested so far cannot compete with respect to Poly(3HA) productivity with the wild-type organisms if the wild-type strains are cultivated under appropriate Poly(3HA)-forming conditions.

However, recombinant *Pseudomonas* strains have been used successfully for the production of Poly(3HA) polymers containing unusual monomers. For example, a Poly(3HA)-negative mutant of *P. putida* KT2442, called GPp109 (Huisman et al., 1991), expressing the Poly(3HA) synthase encoding gene of *Thiocapsa pfennigii* has been cultivated in two-stage batch or fed-batch mode with 5-hydroxyhexanoic acid, 6-hydroxyheptanoic acid, or 8-hydroxyoctanoic acid as a carbon source in the second stage in order to produce polymers containing 5-hydroxyhexanoic acid, 6-hydroxyheptanoic acid, or 8-hydroxyoctanoic acid monomers, respectively (Valentin et al., 1994). A polyester containing Poly(3HA) with 4-hydroxyvaleric acid monomers has been produced in a 15-L scale two-stage aerobic fed-batch process using the recombinant GPp109 strain and octanoic and levulinic acid as carbon sources (Schmack et al., 1998). Cell densities of 20 g L^{-1} could be achieved and the Poly(3HA) content of these cells amounted to up to 50% of cell dry weight. Although the produced polymer consisted mainly of hydroxyhexanoic and hydroxyvaleric acid monomers, the polyesters showed a distinctly elastomeric behavior due to the low content of MCL monomers (15 mol.% hydroxyhexanoic acid and 2 mol.% hydroxyoctanoic acid) (Schmack et al., 1998).

In summary, recombinant *Pseudomonas* strains seem to be useful for the production of polymers containing certain unusual monomers, but less so for the production of the classical MCL-Poly(3HA) polymers.

2.2
Recombinant *E. coli*

E. coli strains blocked in the 3-ketoacyl thiolase (FadA) or 3-hydroxyacyl-CoA dehydrogenase (FadB) enzyme activity of the β-oxidation pathway are able to accumulate MCL-Poly(3HA)s when only the *phaC1* or *phaC2* gene of *Pseudomonas* is expressed (Langenbach et al., 1997; Qi et al., 1998). It is assumed that the β-oxidation has to be slowed down in *E. coli* in order to accumulate specific intermediates, which can serve as precursors for Poly(3HA) synthesis. Various expression systems have been used and, depending on the carbon source and growth conditions Poly(3HA), amounts up to 33% of cell dry weight have been achieved (Table 2). The Poly(3HA) content in these β-oxidation deficient *E. coli* strains could even be further increased to up to 50% of cell dry weight by using acrylic acid, a β-oxidation inhibitor (Qi et al., 1998). Recently, it has been shown that Poly(3HA) can also be produced in *E. coli* strains containing a functional, non-inhibited β-oxidation pathway. Overexpression of a 3-ketoacyl-CoA reductase-encoding gene of *P. aeruginosa* or *E. coli* in addition to one of the Poly(3HA) polymerase encoding genes resulted in the production of 3 to 8% Poly(3HA) per cell dry weight, respectively (Taguchi et al., 1999; Ren et al., 2000b). Moreover, *E. coli* recombinants containing in addition to a Poly(3HA) polymerase a *R*-specific enoyl-CoA hydratase of *P. aeruginosa* with a substrate specificity towards SCL or MCL substrates produced 29 or 3455 Poly(3HA) per cell dry weight, respectively (Tsuge et al., 1999). The existence of *R*-specific enoyl-CoA hydratases in Poly(3HA) producing *Pseudomonas* strains clearly indicates that the Poly(3HA) synthesis pathway proceeds via a stereospecific hydratase reaction rather than the epimerase activity of the β-oxidation. Furthermore, it appears that the monomer composition of the Poly(3HA) produced by the different recombinants is determined by the substrate specificity of the introduced enoyl-CoA hydratase or 3-ketoacyl-CoA reductase (Table 2). Thus, specific *E. coli* recombinants can be engineered in order to produce polymers with desired monomer compositions. In addition, pathway engineering can be used to synthesize MCL-Poly(3HA) with altered physical properties. Introduction of the acetoacetyl-CoA reductase of *Ralstonia eutropha* and blockage of the ketoacyl-CoA degradation step of the β-oxidation not only caused significant changes in the monomer composition but also caused an increase of the molecular weight and loss of the melting point (Ren et al., 2000a). A high molecular-weight peak of around 10^6 Da was observed that could be caused by the higher C6 monomer content of the polymer and which might alter the ratio of chain elongation to chain termination events, resulting in longer Poly(3HA) chains, comparable to Poly(3HB). Another possibility is that the high-molecular weight peak is due to the presence of C6 monomer stretches which facilitate strong non-covalent interactions among Poly(3HA) chains and thus result in the formation of microgels (Ren et al., 2000a).

In summary, it is now possible to produce not only significant amounts of MCL-Poly(3-HA) but also different types of MCL-Poly(3-HA) polymers in recombinant *E. coli*. However, the lack of stability of the recombinants is often a major drawback for the production of sufficient amounts of Poly(3HA) (Ren, 1997). In addition, a major problem in general in applying plasmid-containing recombinants in large-scale fermentation is plasmid maintenance and stability. The classical approach to maintain the phenotype of the recombinant strain is to add antibiotics to the culture medium. This can have a considerable effect on the reproducibility of the results and the final cost of the product. An attractive alternative using minimal sequences for stable, regulated, and inexpensive *phaC* gene expression in recombinant bacteria has been developed by Prieto et al. (1999). The stability of the system to culture MCL-Poly(3HA)-producing recombinant *E. coli* in a bioreactor operated in batch or continuous cultivation mode in the absence of selection marker has been exploited (Prieto et al., 1999). The phenotype was 100% stable throughout the fermentation processes. Furthermore, it has been observed that the chain length of the polymer produced by the recombinants varies depending on the amount of inducer added to the medium. Reduction of inducer concentration caused an increase in the number of polymer molecules with longer chain length, which can only be explained by fewer molecules of Poly(3HA) polymerase (Prieto et al., 1999). This is in agreement with the hypothesis that higher enzyme levels could lead to an increased number of chain initiation events resulting in shorter polymer chain lengths (Haywood et al., 1992).

Taking together all the information gained so far from Poly(3HA) producing recombinant *E. coli* and the advantage that the fermentation and downstream process technology is already established for *E. coli*, it seems likely that *E. coli* is an interesting candidate for the production of specific designed Poly(3HA) polymers in the future.

Tab. 2 Production of MCL-poly(3HA) using recombinant *E. coli* strains

Strain	Genes	Expression system	C source	β-Oxidation inhibition	Percent poly(3HA) (w/w)	Monomer composition (mol.%)					Reference
						C6	C8	C10	C12	C14	
LS1298	*phaC1*$_{Ps}$	P_{tac}	LB, C18	*fadB* mut.	25	HD	4.5	15	21	3.5	Langenbach et al. (1997)
LS1298	*phaC2*$_{Ps}$	P_{tac}	LB, C12	*fadB* mut.	15	ND	6	30	52	38	Qi et al. (1997)
JM109/pJC4	*phaC1*$_{Ps}$	P_{lac}	YE, C16	*fadA* mut.	25	HD	10	90	ND	ND	Ren (1997)
JM109/pJC4	*phaC1*$_{Ps}$	P_{lac}	YE, C16	*fadB* mut.	33	ND	80	20	ND	ND	Ren (1997)
JM109	*phaC1*$_{Ps}$	P_{tac}	LB, C18	acrylic acid	28	HD	ND	ND	ND	ND	Qi et al. (1998)
LS1298	*phaC1*$_{Ps}$	P_{tac}	LB, C18	acrylic acid	50	ND	2.5	30	22	3.5	Qi et al. (1998)
JM109/pJC4	*phaC2*$_{Ps}$ *phaR*$_{Bm}$	P_{lac} P_{tac}	YE, C16	*fadA* mut.	26	ND	42	18	28	ND	Ren et al. (2000a)
JM109/pJC4	*phaC2*$_{Ps}$ *fabG1*$_{Ps}$	P_{lac} P_{tac}	YE, C16	*fadA* mut.	24	ND	23	65	12	HD	Ren et al. (2000b)
RS1318	*phaC1*$_{Ps}$ *fabG*$_{Ps}$	P_{lac} P_{tac}	C8	no	3	ND	9	91	0	HD	Ren et al. (2000b)
LH100	*phaC1*$_{Ps}$ *fabG*$_{Ps}$	P_{lac} P_{tac}	C12	no	8	0	12	80	8	0	Taguchi et al. (1999)
LS3298	*phaC1*$_{Ps}$ *fadI*$_{Ec}$	P_{lac} S_{tac}	C12	no	29	10	28	7	3	2	Tsuge et al. (1999)
LS1298	*phaC1*$_{Ps}$ *fadI*$_{Ec}$	P_{lac} S_{tac}	C12	no	14	8	35	23	11	6	Tsuge et al. (1999)

3H: 3-Hydroxyhexanoate; C8: 3-hydroxyoctanoate; C10: 3-hydroxydecanoate; C12: 3-hydroxydodecanoate; Ps: *P. aeruginosa*; Pse: *P. oleovorans*; Re: *R. eutropha*; Ec: *E. coli*; LB: Luria Bertani medium; YE: Yeast Extract; ND: not determined.

8
Downstream Processing

Recovery procedures for MCL-Poly{3HA} resemble those originally developed for the production of Poly{3HB}. A number of solvent extraction processes have been assessed to separate MCL-Poly{3HA}s from biomass. These usually involve the use of a chlorinated solvent such as chloroform (Lageveen et al., 1988) or methylene chloride. Recently, it has been reported that MCL-Poly{3HA}s can be extracted with hexane or acetone instead of chlorinated solvents (Williams et al., 1999) and subsequently precipitated by the addition of a non-solvent for the Poly{3HA}, such as methanol. Using this method, the resulting polymer can be obtained in high purity. An alternative, non-solvent-based extraction process was developed by de Koning et al. (1995a,b) and further optimized to make the overall production process more attractive (Reitsema, 1996). The biomass is separated from the medium by centrifugation and treated with a protease cocktail and a detergent to solubilize all cell components. Removal of the solubilized cell material and concentration of the resulting Poly{3HA} suspension is achieved by crossflow microfiltration (Reitsema, 1996) or continuous centrifugation (unpublished results). The subsequent MCL-Poly{3HA} granules display a density close to that of water (Preusting et al., 1993), as a result of which a MCL-Poly{3HA} suspension does not settle (Marchessault et al., 1995). In fact, it forms a highly stable polymer latex. The overall purity of the latex amounts to 95%. Furthermore, supercritical CO_2 is highly effective at extracting lipids and other hydrophobic contaminants from Poly{3HA}-containing cells and 100% purity can be reached in a single step (Williams et al., 1999).

Since the liberation of chromosomal DNA during lysis causes a dramatic increase in viscosity, a nuclease encoding gene from *Staphylococcus aureus* was integrated into the genomes of several Poly{3HA} producers. The nuclease is directed to the periplasm, and occasionally to the culture medium, without affecting Poly{3HA} production or strain stability, and reducing the viscosity of the lysate significantly during the downstream process (Brayton et al., 1999).

9
Production

MCL-Poly{3HA} has, in contrast to Poly-{3HB}, not been produced on a commercial scale yet. There is sufficient material available for R&D purposes and several applications have been developed.

9.1
MCL-Poly{3HA} Production versus SCL-Poly{3HA} Production

In contrast to Poly{3HB}, MCL-Poly{3HA} has not been produced on a commercial scale yet. The process development of Poly-{3HB} has also received a lot more attention than processes for the production of MCL-Poly{3HA}. It is therefore interesting to compare production parameters of MCL-Poly{3HA} production with those of Poly-{3HB}. The parameters of the best Poly-{3HB} and MCL-Poly{3HA} processes are given in Table 3.

Looking at the fed-batch operated cultures the main difference concerning process parameters between Poly{3HB} and MCL-Poly{3HA} production seems to be the lower MCL-Poly{3HA} content. It is reported that a low MCL-Poly{3HA} content decreases the productivity and yield, and increases the costs for down-stream processing and waste disposal (Choi et al., 1999).

Tab. 3 Process parameters of poly{3HB} and MCL-poly{3HA} production

	Poly{3HB}	*MCL-poly{3HA}*	
Organism	*A. latus*	*P. putida*	*P. oleovorans*
Fermentation type	fed-batch	fed-batch	two-stage continuous
Substrate	sucrose	coconut oil fatty acids	octane
Culture time (h)	20	36	–
Cell concentration (g L^{-1})	111.7	133	57
Poly{3HA} content (%)	88	59	65
Productivity (g L^{-1} h^{-1})	4.78	2.3	1.86
Yield (g g^{-1})	0.42	0.5–0.4	not determined
Reference	Wang and Lee (1997)	see Figure 2	Huisenveld (1992)

Notable, the Poly{3HA} content is always expressed as the weight ratio between Poly-{3HA} and total biomass weight. The density of Poly{3-HB}, however, is 1.24 g mL^{-1} (Marchessault et al., 1990) whereas the density of MCL-Poly{3HA}, depending on the monomer composition, is close to 1.00 g mL^{-1}. On a volume basis, the Poly{3HA} content of 65% (Table 3) in *P. oleovorans* corresponds with a Poly{3HB} content of 82%! Also, in terms of applications, the volume of the material is more important than the weight. If Poly{3HB} and MCL-Poly{3HA} could be used for the same application, 24% more Poly{3HB} would be necessary on a weight basis.

9.2
Producers

MCL-Poly{3HA} is not produced at a commercial scale yet. It is being produced routinely at ATO (The Netherlands) with *P. putida* KT2442 using fatty acids as substrate. Typical fermentation process parameters are: 120 g L^{-1} biomass containing 50% MCL-Poly{3HA} produced in 24–35 h. MCL-Poly{3HA} batches (several kilogram amounts) with specific monomer compositions are available for research purposes.

9.3
Applications

The application of MCL-Poly{3HA} has been reviewed extensively by van der Walle et al. (2001).

The material properties of MCL-Poly{3-HA} are strongly related to the chemical characteristics (i.e. monomer composition) of the various polymers. Since the polyester structure can be tailored quite simply, the polymer properties therefore can be readily adjusted to meet the specific demands for a particular application. Moreover, the unsaturated MCL-Poly{3HA}s are chemically reactive and completely amorphous.

MCL-Poly{3HA}s can be manufactured to many different materials and shapes. Furthermore, they can be processed in latex (granules in water) or in solution with several different solvents. Together with the material properties of MCL-Poly{3HA}s, this opens up a whole field of feasible commercial applications to be explored and exploited.

In general, due to their biodegradability, water resistance, and oxygen impermeability, Poly{3HA}s can be used for all sorts of biodegradable packaging materials, including composting bags and food packaging. Also, the use of Poly{3HA}s in single-use sanitary articles like diapers is considered as

economically feasible. In addition, in marine environments (fishing nets and other discarded objects that cause severe damage when made from non-degradable materials), construction materials (adhesives, laminates, foams and rubbers), and in agricultural industries, there is promising market potential for new biodegradable materials.

The potential for biomedical applications is very promising, since the added value to these special products is remarkably high (Hocking and Marchessault, 1994; Lafferty et al., 1988; Williams et al., 1999); although research in this field is of unique complexity, it is both technical and economical very rewarding to succeed.

Several applications on basis of MCL-Poly(3HA) have been developed.

9.3.1

Pressure-sensitive Adhesives (PSAs)

Babinec et al. (1992) described the development of a biodegradable PSA on the basis of MCL-Poly(3HA)s. Different Poly(3HA)s were tested, produced by cultivating *P. oleovorans* on octanoic acid, decanoic acid, mixtures of octanoic and nonanoic, or mixtures of octanoic and 10-undecenoic acid. Tackifiers were added to the Poly(3HA) to give a PSA with improved tack and the strength of the Poly(3HA) was increased by UV radiation crosslinking using a photosensitizer. All but the mixtures with octanoic acid gave PSAs with good properties. Biodegradation studies indicated that the PSA formulations were still biodegradable (Babinec et al., 1992).

9.3.2

Biodegradable Rubbers

Biodegradable rubbers have been manufactured from unsaturated Poly(3HA)s, by crosslinking of the biopolyesters. This has been accomplished by either chemical reaction with sulfur or peroxides (Gagnon et al., 1994a,b), or by radiation curing using UV or an electron-beam source (De Koning et al., 1994; Ashby et al., 1998). The MCL-Poly(3-HA)-based rubbers are still biodegradable because the ester bond is still hydrolyzable. By choosing different types of starting material and varying the crosslinking conditions, material properties like mechanical strength, tear resistance, tensile set, and flexibility of the biorubbers were readily adjusted (De Koning et al., 1994; Gagnon et al., 1994a,b; Ashby et al., 1998).

9.3.3

Paint Binders

Recently, the development of environmentally friendly paints and coatings based on MCL-Poly(3HA) has been reported (van der Walle et al., 1999). Fatty acid mixtures derived from tall oil, linseed oil, and rape seed oil with unsaturated fatty acids have been used as a substrate for MCL-Poly(3HA) paint binders. Due to the relatively low molecular weight and narrow molecular weight distribution of MCL-Poly(3HA), the viscosity of the resulting paint is low compared to synthetic binders such as polyacrylates and polyurethanes. To adjust the viscosity of the MCL-Poly(3HA) paint to optimal values for paint applications, less organic solvents are necessary compared to the synthetic binders. This could have a significant potential, since organic solvents in DIY paints will be, and in some EU countries already are, further restricted by future legislation. Further studies are focused on the application of MCL-Poly(3-HA) latexes in totally organic solvent free paints. The application of such water borne paint systems is a promising perspective in further reducing the use of organic solvents in paints and coatings (van de Walle et al., 1999).

9.3.4

Cheese Coatings

Cheeses are generally coated by a non-biodegradable, synthetic plastic-based latex, typically a copolymer of polyvinyl acetate and dibutyl maleic acid. This has prompted research towards the development of a fully biodegradable cheese coating.

The technical demands for a cheese coating are very comprehensive since it has to fulfill a large number of functions (Castle et al., 1993), such as mechanical and hygienic protection, semi-permeability for water, CO_2 and certain other flavoring components, easy applicability, long stability, etc.

A new biodegradable cheese coating has been developed on the basis of a MCL-Poly(3HA) latex derived from saturated fatty acids. An extensive test program showed that the functional aspects of the Poly(3HA)-based cheese coatings, like ripening control and mechanical and bacterial protection, are equivalent to the current generation of plastic coatings (van der Walle et al., 2001).

9.4

Patents

There are many patents concerning Poly(3-HA)s in general; many of them also valid for MCL-Poly(3HA)s. There are only of few patents specifically for microbial MCL-Poly-(3HA) production and applications on the basis of MCL Poly(3HA)s (Table 4).

There are two key patents on the fermentative production of MCL-Poly(3HA) and its monomers. In WO9831824A1 (Witholt et al., 1997) the production of MCL-Poly(3-HA) and its monomers by fluorescent Pseudomonads from aliphatic substrates is claimed. The production of MCL Poly(3HA) and its monomers by transformed *E. coli* is claimed in WO9854329 (Witholt et al., 1998).

The applications mentioned above (medical applications, paints, cheese coatings and adhesives) are patented (Table 4).

10

Outlook and Perspectives

MCL-Poly(3HA) is a unique (bio)polymer due to such properties as biodegradability, biocompatibility, water insensitivity, and chemical reactivity. Due to these characteristics MCL-Poly(3HA)s have their own niche in application development.

MCL-Poly(3HA) is not one polymer, but a class of biopolyesters. The monomeric composition is variable and can be easily controlled by simply changing the aliphatic fermentation feedstock. In this way it is possible to produce a whole range of bioplastics with distinctive material properties, allowing the tailoring of the material characteristics to meet the demands of several applications. This increases the applicability of MCL-Poly(3HA); it cannot only be used for bulk applications but also for specialties. Different types of MCL-Poly(3HA) can all be produced using the same or similar fermentation process by simply changing the type of substrate(s) used. In that way it is possible to produce tailor-made MCL-Poly(3HA) variants for specific applications – in other words, it is possible to produce high added-value specialties at a low cost, bulk scale.

The costs of fermentative MCL-Poly(3HA) production are mainly caused by costs for feedstock, but also for a significant part by costs for waste disposal and cooling. Further optimization of MCL-Poly(3HA) fermentation processes has to focus on these three items. A further increase in MCL-Poly(3HA) content of the microbial biomass is the best solution, since it will decrease costs for feedstock, downstream processing, cooling,

Tab. 9 Patents concerning fermentation processes and applications specific for MCL-poly(3HA)

Number	Holder	Inventors	Title	Date of publication
WO9301281	Rijksuniversiteit Groningen	Witholt, B., Eggink, G., Huisman, G.W.	Microbiological production of polyesters	October 1990
JP06081239	Yamaha Corp.	Ishikawa Kenji	Medical soft member	January 1993
JP06038759	Fuso Steel Ltd.	Maedakenen, H., Morio, M., Yoshimasa, F.	Microorganism capable of producing poly(3-hydroxyalkanoate)	February 1994
WO9404685	Stichting Dienstverlening en Onderzoek Noord Nederland (SDON)	Eggink, G. and Hoekstra, M. D.	Method for the production of biologically degradable polyhydroxyalkanoate coating with the aid of an aqueous dispersion of polyhydroxyalkanoate	January 1996
US5618775	Minnesota Mining & Manufacturing Co.	Sutherland, D. R., Hanson, J., Rides, G. N.	Poly(3-hydroxyoctanoate) pressure sensitive adhesive compositions	March 1997
WO9905182	Metabolix Inc.	Williams, S. F., Martin, D. P., Gramgeorge, T., Horowitz, D. M.	Polyhydroxyalkanoates for in vivo applications	November 1998
JP63051291	ICI	Kessler, B., Witholt, B.	Production of medium chain length poly-3-hydroxyalkanoates in *E. coli*, and microorganisms thereof therefore	December 1988
WO9959588	W.M. Wrigley Jr. Co.	G. W. Orlan, C. Du, L. Evans, J.W.	Environmentally friendly chewing gum boxes including polyhydroxyalkanoates	August 1999
US6329181	Institut for Agrobiotechnological Research (ATO)	Steinbuss, G. S. H., Cuperus, F. P., Windhuis, R. A., Eggink, G.	Poly(3-hydroxyalkanoate) plant and method for the preparation thereof	February 2001

soil waste disposal simultaneously (Choi and Lee, 1999).

An alternative for fermentative production of MCL-Poly(3HA) is by means of genetically engineered plants (Poirier, 1999; Van der Leij and Witholt, 1995). The production of MCL-Poly(3HA) by *Arabidopsis thaliana* has been investigated (Mittendorf et al., 1998). A polymer content of 0.4% has been reached. The time to market of these materials is estimated at 10–15 years from now. The production costs of MCL-Poly(3HA) in plants is potentially lower than when MCL-Poly(3HA) is produced in fermentation processes. It is likely that the flexibility to incorporate various monomers will decrease when genetically modified plants are used for MCL-Poly(3HA) production. Even then, however, the possibility to (bio)chemically modify the polymers makes it possible to adapt the material characteristics to meet the demands of a multitude of applications.

MCL-Poly(3HA) is not available on the market yet. There are two main reasons to consider.

First of all, the economics of fermentative MCL-Poly(3HA) production is often compared with that of SCL-Poly(3HA). However, this is not justified since these are completely different materials. SCL-Poly(3HA)s have to compete with commodity plastics such as polyethylene and polypropylene. The costs of these commodity plastics are so low (0.96–1.10 and 0.84 $/kg, respectively; *Chemical Market Reporter*, January 2001) that fermentatively produced SCL-Poly(3HA) will not be able to compete. MCL-Poly(3HA), on the other hand, as a specialty polymer, has to compete with materials such as polyurethanes, isoprenes, styrene butadienes, and chloroprenes. The price of these materials varies between 2–5 $ kg^{-1}. In a state-of-the-art fermentation process MCL-Poly(3-HA) can be produced with production costs ranging between 3.5 and 6.5 $/kg MCL-Poly(3HA), indicating that fermentatively produced MCL-Poly(3HA) indeed could compete cost-wise with its synthetic counterparts.

Secondly, the obvious advantage that the material characteristics of MCL-Poly(3HA) can be programmed also has an important negative side-effect. In order to adjust the material characteristics of MCL-Poly(3HA) to meet the demands of the application, the application developer has to work in close cooperation with the MCL-Poly(3HA) producer. This also implies that a potential MCL-Poly(3HA) producer has to have a wide network of application developers, to establish a sufficient market for bulk-scale production of MCL-Poly(3HA). On the other hand, this reduces the risk for the producer since its products are used for several applications and bought by several clients.

To introduce MCL-Poly(3HA) on the market in a short term it is therefore important to establish a network of (a) potential MCL-Poly(3HA) producer(s) and application developers and the availability of significant amounts of tailor-made MCL-Poly(3HA) to allow small-scale application development, field trials, and market introduction of specific high-end MCL-Poly(3HA) products.

Acknowledgements

Parts of the research presented in this chapter was performed in an EET project (EETK96088) sponsored by the Dutch Ministries of Economische Zaken (EZ), Onderwijs, Cultuur en Wetenschappen (OCenW), and Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer (VROM).

11
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11
Biosynthesis and Fermentative Production of SCL-MCL-PHAs

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